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Interaction of the PAS B Domain with HSP90 Accelerates Hypoxia-Inducible Factor-1α Stabilization

Dörthe M. Katschinski[1], Lu Le[2], Susann G. Schindler[2], Tim Thomas[3], Anne K. Voss[3] and Roland H. Wenger[4]

[1]Cell Physiology Group, Medical Faculty, Martin-Luther-University Halle, Germany, [2]Institute of Physiology, University of Lübeck, Germany, [3]The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia, [4]Institute of Physiology, University of Zürich, Switzerland

Key Words

Chaperone • Geldanamycin • Gene regulation • Hyperthermia • Oxygen • Prolyl hydroxylation • Proteasome

Abstract

Hypoxia-inducible factor (HIF) α subunits are induced under hypoxic conditions, when limited oxygen supply prevents prolyl hydroxylation-dependent binding of the ubiquitin ligase pVHL and subsequent proteasomal degradation. A short normoxic half-life of HIF-α and a very rapid hypoxic protein stabilization are crucial to the cellular adaptation to changing oxygen supply. However, the molecular requirements for the unusually rapid mechanisms of protein synthesis, folding and nuclear translocation are not well understood. We and others previously found that the chaperone heat-shock protein 90 (HSP90) can interact with HIF-1α *in vitro*. Here we show that HSP90 also interacts with HIF-2α and HIF-3α, suggesting a general involvement of HSP90 in HIF-α stabilization. The PAS B domain, common to all three α subunits, was required for HSP90 interaction. ARNT competed with HSP90 for binding to the PAS B domain since an excess of either component inhibited the activity of the other. HSP90 as well as the heterocomplex members HSP70 and p23, but not HSP40, were detected in immunoprecipitations of endogenous cellular HIF-1α. While HSP90 and HSP70 bound to HIF-1α predominantly under normoxic conditions, ARNT bound to HIF-1α primarily under hypoxic conditions, suggesting that ARNT displaced HSP90 from HIF-1α following nuclear translocation. Hypoxic accumulation of HIF-1α was delayed in a novel cell model deficient for HSP90β as well as after treatment of wild-type cells with the HSP90 inhibitor geldanamycin, suggesting that HSP90 activity is involved in the rapid HIF-1α protein induction.

Introduction

The hypoxia inducible transcription factor-1 (HIF-1) is an important regulator of the cellular response to hypoxia [1, 2]. HIF-1 comprises two subunits, the oxygen labile α-subunit and the constitutively expressed β-subunit aryl hydrocarbon receptor nuclear translocator (ARNT) [3]. HIF-1α is modified by oxygen-dependent prolyl hydroxylation, allowing the binding of the von Hippel-Lindau tumor suppressor protein (pVHL) that targets HIF-

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PD. Dr. med. D.M. Katschinski
Cell Physiology Group, Martin-Luther-University Halle
Magdeburgerstrasse 2, D-06112 Halle (Germany)
Tel. +49-(0)345 557 1374, Fax +49-(0)345 557 1378
E-Mail doerthe.katschinski@medizin.uni-halle.de

1α for ubiquitinylation and proteasomal destruction [4-7]. Limited oxygen supply prevents hydroxylation and thus degradation. This unusual mechanism of protein regulation provides the basis for the extremely rapid HIF-1α response to hypoxia which apparently occurs without any measurable delay [8].

Activation of HIF-1α also involves nuclear translocation, dimerization with ARNT, DNA-binding and recruitment of transcriptional co-activators. These processes can be triggered by post-translational modifications of HIF-1α. It has been demonstrated that activation of the PI3-kinase-PTEN-Akt-GSK3/FRAP and the Ras/Raf-MEK-p42/44 kinase pathways eventually result in elevated HIF-1α protein levels and/or transcriptional activity of HIF-1 [9-11]. Under normoxic conditions, HIF-1α is further modified by asparagine hydroxylation, preventing the recruitment of transcriptional co-activators [12-14]; and by lysine acetylation, promoting pVHL binding [15]. Apart from HIF-1α, two other genes encoding for the hypoxia-inducible factors HIF-2α/EPAS1 and HIF-3α (including its splice variant IPAS), respectively, have been reported [16-20]. All three α-subunits share many similarities in terms of stabilization and heterodimerization with ARNT.

Previous reports indicated that HIF-1α can bind to the heat shock protein 90 (HSP90) *in vitro* [21, 22] or in cells transfected with a fusion protein [23]. HSP90-binding prevents degradation of many cellular proteins including basic-helix-loop-helix (bHLH) transcription factors such as the dioxin receptor and steroid hormone receptors, p53 and protein kinases [24, 25]. In case of the steroid hormone receptor, HSP90-binding also involves HSP70, HSP40 as well as the co-chaperone p23 and several immunophilins. The composition of this heterocomplex is highly dynamic due to ATP-dependent cycling. Upon exposure to heat, ATP-dependent stabilization has been described for some of the HSP90 client proteins [24, 25].

In a prior study, we demonstrated that heat is also sufficient to stabilize the unphosphorylated form of HIF-1α under normoxic conditions, which was mediated by HSP90-binding [26]. In addition, we and others found that inhibition of HSP90 activity with geldanamycin, novobiocin or radicicol prevents not only heat-induced but also hypoxia-induced stabilization of HIF-1α [23, 26-28]. Antibiotics of the ansamycin family such as geldanamycin and novobiocin might thus be useful as small molecule drug inhibitors of HIF-1α overexpression in cancer cells [29-31]. Disruption of the HIF-1α-pVHL interaction in pVHL-deficient cells or by prolyl hydroxylation-defective HIF-1α mutants, did not affect geldanamycin-induced proteasomal degradation of HIF-1α [32]. These results suggest that Hsp90 protects HIF-1α from oxygen-independent degradation by an unidentified HIF-1α ubiquitin ligase.

Thus, HIF-1α might represent a novel HSP90 client protein and HSP90 appears to regulate HIF-1α stabilization. However, it can not be formally ruled out that HSP90 antagonists inhibit HIF-1α stabilization by unspecific mechanisms independent of HSP90 inhibition. Therefore, we examined the hypoxic stabilization of HIF-1α in a novel embryonic stem (ES) cell model established from mouse blastocysts containing two targeted alleles of the gene encoding HSP90β [33]. To further elucidate the mechanism by which HSP90 regulates HIF-1α, we investigated the protein-protein interaction with HSP90 *in vitro* and *in vivo* and we determined the HIF-1α domain responsible for the interaction with HSP90.

Materials and Methods

Antibodies and Chemicals

Antibodies were purchased from the suppliers indicated in parentheses: mouse monoclonal IgMκ anti-HSP90 (Affinity Bioreagents), polyclonal rabbit anti-HSP90α (StressGen), mouse monoclonal anti-HSP90 (StressGen), mouse anti-HIF-1α (Novus), mouse anti-HSP70 (Alexis), rabbit anti-HSP40 (Calbiochem), mouse anti-p23 (Affinity Bioreagents), mouse anti-β-actin (Sigma), control mouse IgG_2 (Dako). Appropriate horseradish peroxidase-labeled secondary antibodies were purchased from SantaCruz and Promega. Geldanamycin was purchased from Alexis. All other chemicals were obtained from Sigma.

Plasmid constructions

The plasmid pBSKShuHSP90 was obtained from American Type Culture Collection (ATCC) and contains the full-length cDNA of human HSP90 which was subcloned into pcDNA3 (InvitroGen). Plasmids containing full length cDNAs for HIF-1α, HIF-2α HIF-3α, ARNT and p23 were kindly provided by G. L. Semenza (Baltimore, MD, USA), Y. Fujii-Kuriyama (Osaka, Japan), C. A. Bradfield (Madison, WI, USA), L. Poellinger (Stockholm, Sweden) and M. Garabedian (New York, NY, USA), respectively. The PAS B domain of HIF-1α was deleted by digestion with *Bsp*MI and *Afl*II (MBI Fermentas), blunting with Klenow polymerase and re-ligation, resulting in the loss of amino acids 222 to 390 of HIF-1α [3].

Cell culture

Human HepG2 hepatoma cells were obtained from ATCC. Cells were grown in high-glucose DMEM containing 10% FCS (LifeTechnologies) in a humidified 5% CO_2/95% air atmosphere at 37°C. ES cells homozygous for an HSP90β null allele were

isolated from blastocysts recovered from heterozygous HSP90β intercrosses [33] as described previously [34]. ES cells were cultured in high-glucose DMEM containing 20% FCS and 10^6 U LIF/l. Immortalized and transformed mouse embryonic fibroblasts (MEFs) derived from wild-type and HIF-1α-deficient mice were described previously [35]. For hypoxic exposure, the O_2 concentration in the cell culture incubator ($InVivO_2$, Ruskinn, Leeds, UK) was lowered to 1%.

Protein extraction and immunoblot analyses

Following stimulation, cells were collected, washed with ice-cold PBS and extracted with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 400 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF. Protein concentrations were determined using the Bradford method. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes by semi-dry electroblotting (Bio-Rad). Membranes were stained with Ponceau S to verify equal protein loading per lane. After overnight blocking (5% non-fat milk powder in PBS), the blots were incubated with the primary and secondary antibodies mentioned above. Chemiluminescence detection was performed by incubating the membranes with 100 mM Tris-HCl (pH 8.5), 2.65 mM H_2O_2, 0.45 mM luminol and 0.625 mM coumaric acid for 1 min followed by exposure to X-ray films.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described before [26]. Oligonucleotides containing a HIF-1 DNA-binding site derived from the erythropoietin gene were gel-purified on 10% polyacrylamide gels prior to 5' end-labeling of the sense strand with γ^{32}P-ATP (Amersham). Unincorporated nucleotides were removed by gel filtration over Bio-Gel P60 columns (Bio-Rad). Labelled sense strands were annealed to a twofold molar excess of unlabelled antisense strands. DNA-protein binding reactions were carried out for 4 hours at 4°C in a total volume of 20 μl 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol, containing 5 μg nuclear extract, 0.1 μg sonicated calf thymus DNA (Sigma), and 2 x 10^4 cpm of oligonucleotide probe. Samples were run on 4% nondenaturing polyacrylamide gels at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4°C. The gels were dried and radioactive signals were recorded by phosphoimaging.

Co-immunoprecipitation with in vitro *translated proteins*

Coupled *in vitro* transcription and translation (IVTT) was performed with rabbit reticulocyte or wheat germ lysates according to the manufacturer's recommendations (Promega). HSP90 was translated in rabbit reticulocyte lysates and wheat germ lysates were used for all other proteins. IVTTs (20 μl of each reaction) were incubated for 2 hours at 4°C in a total volume of 500 μl of MENG buffer (25 mM MOPS pH 7.5, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 20 mM sodium molybdate, 10 mM DTT). Subsequently, antibodies were added and incubated for another 90 min. Finally, 100 μl of a 50% proteinGplus/proteinA agarose slurry in MENG buffer containing 5 mg/ml albumin were added for 90 minutes. Agarose beads were washed three times with MENG buffer containing 2 mM sodium molybdate. Immunoprecipitated proteins were recovered by adding 0.5 M Tris-HCl (pH 6.8), 0.5% SDS, 0.05% bromophenol blue, 20% glycerol. Samples were cleared from agarose beads by filtration through micropure 0.22 separators (Amicon), boiled and separated by SDS-PAGE. The gels were fixed in 50% methanol/10% acetic acid, dried and the radioactive signals were recorded by phosphoimaging.

Co-immunoprecipitation with cellular protein extracts

Protein extracts (2 mg) were precleared with 100 μl ProteinGplus/ProteinA agarose (Oncogene) in extraction buffer containing 5 mg/ml bovine serum albumin. Subsequently, the samples were incubated with anti HIF-1α (Novus) antibodies for 2 hours. Protein Gplus/A agarose (100 μl) was added and incubated overnight. The beads were washed, precipitated and recovered as above. HIF-1α, HSP90, HSP70, HSP40 and p23 were detected by immunoblot analyses as described above.

Reporter gene assays

HepG2 cells were transiently co-transfected by electroporation with expression vectors, a hypoxia-responsive luciferase reporter gene (pH3SVL) and a constitutive renilla expression vector (pRL-SV40) as described previously [36]. The expression vector pcDNA3 was either empty or contained HSP90 or p23 full-length cDNAs (see above). The luciferase reporter gene pH3SVL was driven by a SV40 promoter and contained a total of 6 HIF-1 DNA-binding sites derived from the transferrin gene [37]. Following transfection, the cells were split and exposed to normoxic or hypoxic conditions. Reporter gene activities were determined as described by the manufacturer (Promega). Results were normalized to the values obtained with the constitutive renilla reporter gene and are expressed as hypoxia to normoxia ratios.

Results

All three HIF-α subunits interact with HSP90 in vitro

To investigate the interaction between HIF-α subunits and HSP90, co-immunoprecipitation assays of *in vitro* translated proteins were performed. Radioactively labeled HIF-1α (Fig. 1A) but not ARNT (Fig. 1B) co-precipitated with HSP90. The specificity of this protein-protein interaction was confirmed by the presence of radioactively labeled HSP90 in HIF-1α precipitates (Fig. 1C). Similar results were obtained with radioactively labeled HIF-2α and HIF-3α which co-precipitated with HSP90 (Fig. 1D), suggesting that a domain common to all three HIF-α subunits serves as the HSP90 interaction interface.

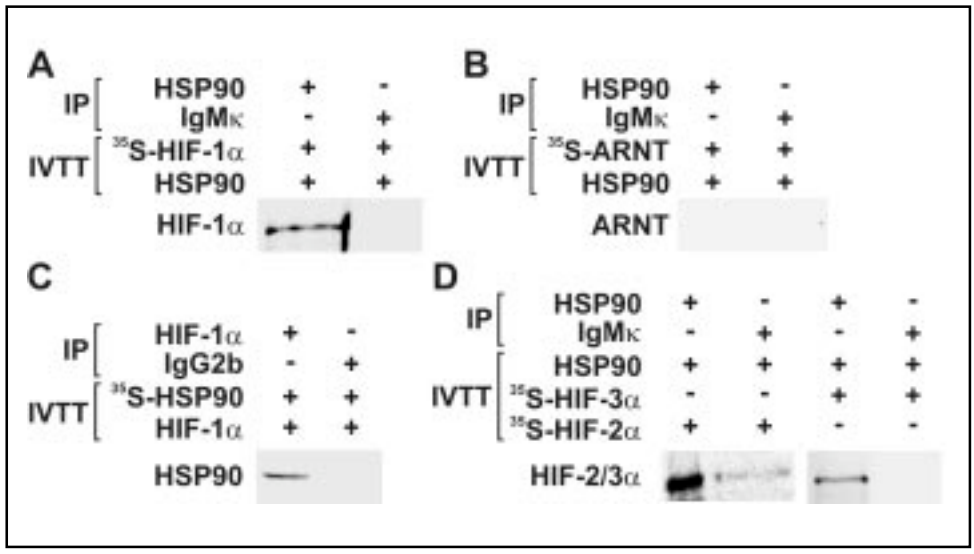

Fig. 1. Interaction of HIF subunits with HSP90. *In vitro* transcription/translation (IVTT) followed by immuno-precipitation (IP) of HSP90 (A, B, D) or HIF-1α (C). Either HIF-1α (A), ARNT (B), HSP90 (C), HIF-2α (D, left) or HIF-3α (D, right) were radioactively marked by incorporation of ^{35}S-labeled amino acids in IVTT reactions. The antibodies used for immunoprecipitation were derived against the non-labeled interacting protein. IgMκ (A, B, D) and IgG_{2b} (C) isotype-matched antibodies were used for negative control immuno-precipitations. Shown are phosphoimaging pictures of washed co-immunoprecipitates separated by SDS-PAGE.

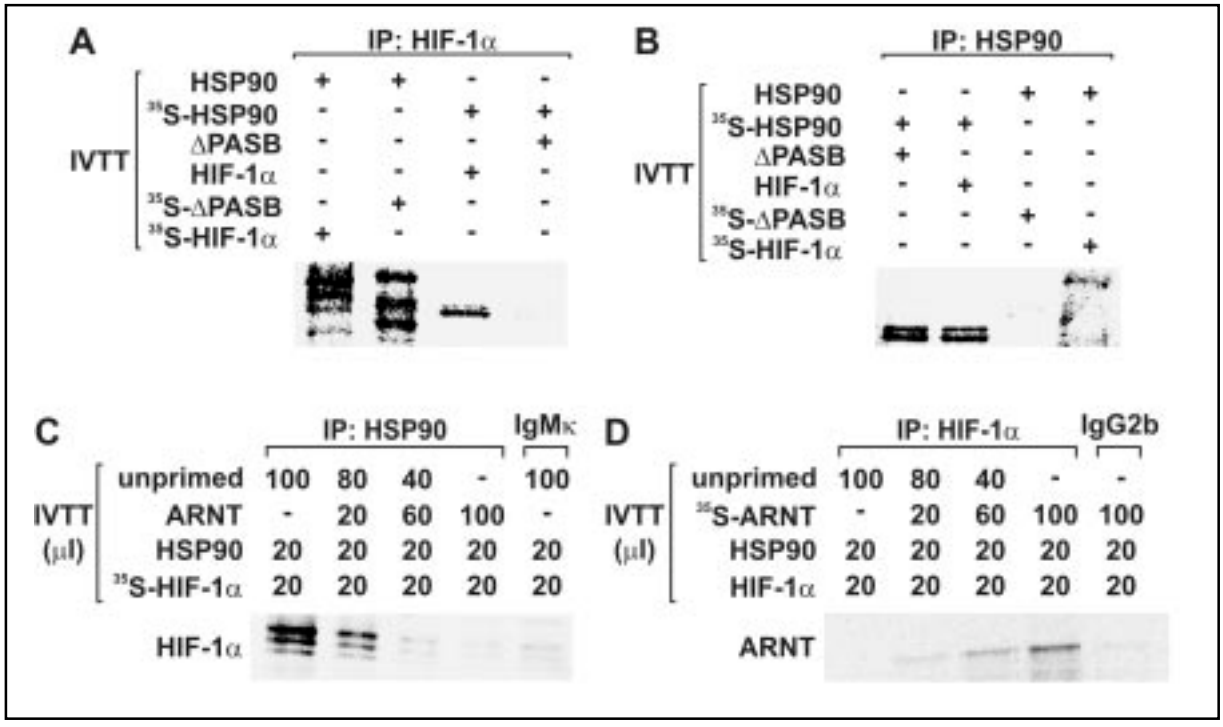

Fig. 2. Inhibition of HIF-1α-HSP90 interaction by deletion of the HIF-1α PAS B domain or addition of ARNT. IVTT followed by IP of HIF-1α (A) or HSP90 (B). Increasing amounts of ARNT, produced in a separate IVTT reaction, were added to HSP90-HIF-1α interaction assays, followed by immunoprecipitation (IP) of HSP90 (C) or HIF-1α (D). Either HSP90, wild-type HIF-1α, PAS B-deleted HIF-1α (ΔPASB) or ARNT were radioactively marked by incorporation of ^{35}S-labeled amino acids in IVTT reactions. IgMκ (C) and IgG_{2b} (D) isotype-matched antibodies were used for negative control immuno-precipitations. To equalize the total volume of lysate in the reaction, the indicated amounts of unprimed reticulocyte lysate devoid of any plasmid were added. Note that some fragmentation of the labile HIF-1α and ΔPASB proteins occured in these experiments, which, however, did not affect the general conclusions.

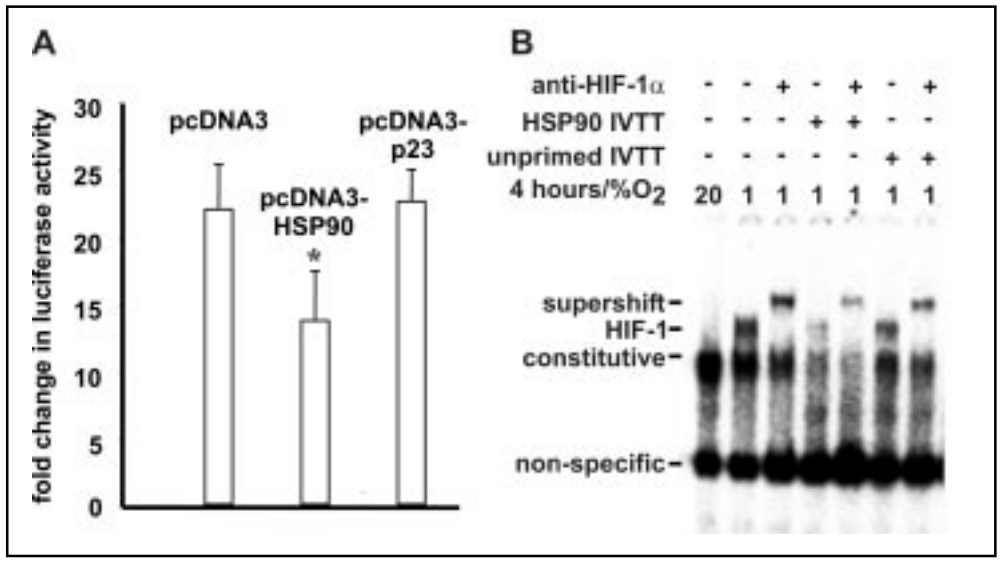

Fig. 3. Inhibition of HIF-1-dependent reporter gene induction and DNA-binding by HSP90. A Hypoxic induction factors of a HIF-1-dependent luciferase reporter gene transiently transfected into HepG2 cells. The pcDNA3 expression vector, containing or not containing HSP90 and p23 cDNAs, was co-transfected together with the firefly luciferase reporter gene and a constitutive renilla luciferase expression vector. Shown are means ± s.d. of relative values normalized to the corresponding renilla activities. *, $p<0.05$, n=3. B EMSA of nuclear extracts derived from HepG2 kept for 4 hours at 20% or 1% O_2. Supershift analysis with a monoclonal anti-HIF-1α antibody confirmed the specificity of the HIF-1 bands. HSP90-primed or unprimed IVTT reactions were added to HIF-1 DNA-binding reactions.

The PAS B domain is required for HSP90 interaction

The Per-ARNT-Sim (PAS) domain is an approx. 300 amino acids segment, which can be subdivided into two copies of approx. 50 amino acids degenerate repeats called the PAS A and PAS B domains. In the PAS family of mammalian transcription factors, PAS domains function as heterotypic and probably also homotypic interaction interfaces. The PAS B domain of the dioxin receptor is also a well-known HSP90 interaction domain. We thus deleted the PAS B domain of HIF-1α, resulting in ΔPAS B. Immunoprecipitation of radioactively labeled ΔPAS B demonstrated that antibody recognition was not affected by the lack of the PAS B domain (Fig. 2A). However, HSP90 was almost completely lost in the ΔPAS B precipitates (Fig. 2A). This was further substantiated by the presence of radioactively labeled HIF-1α but not ΔPAS B in HSP90 precipitates (Fig. 2B).

ARNT competes with HSP90 for HIF-1α binding

Because the PAS B domain of HIF-1α is also involved in the interaction with ARNT, we determined whether HSP90 and ARNT compete for binding to HIF-1α. Therefore, increasing amounts of ARNT were added to a HSP90-HIF-1α protein-protein interaction assay. As shown in Fig. 2C, ARNT decreased the amount of radioactively labeled HIF-1α in HSP90 precipitates, suggesting that ARNT prevents HIF-1α-HSP90 interaction. Instead, increasing amounts of radioactively labeled ARNT were found in HIF-1α precipitates (Fig. 2D), confirming that ARNT displaced HSP90 from the HIF-1α binding site. Taken together, these data indicate that binding of either ARNT or HSP90 to HIF-1α is mutually exclusive.

HSP90 suppresses HIF-1 transactivation and DNA-binding activity

To further investigate the model of HSP90-ARNT competition for HIF-1α binding, we next determined the impact of HSP90 dysregulation on HIF-1 function. Therefore, HSP90 was overexpressed in HepG2 cells by transient transfection and the influence of HSP90 on endogenous HIF-1 transactivation activity was analyzed. HIF-1 function was assessed by measuring the activity of a co-transfected luciferase reporter gene under the control of a total of six HIF-1 DNA-binding sites derived from the transferrin gene [37]. Exposing transiently transfected cells to hypoxia for 24 hours resulted in an induction of about 20-fold when compared to normoxic cells (Fig. 3A). Simultaneous transfection of a HSP90 expression vector, but not the empty expression vector or a co-chaperone p23 expression vector, resulted in a significant ($p<0.05$, n=3) decrease of the hypoxic reporter gene induction (Fig. 3A).

To determine the influence of overtitrated HSP90 on HIF-1 DNA-binding, EMSAs were performed

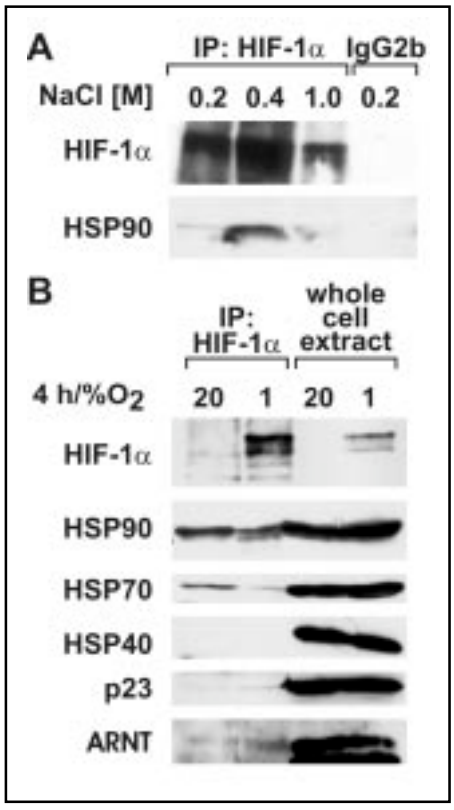

Fig. 4. Cellular interaction of HIF-1α with the HSP90 heterocomplex. A Immunoblot analysis of HIF-1α and HSP90 in nuclear extracts derived from HepG2 cells kept for 4 hours at 1% O_2. Cells were lysed with NP-40 and extracted with increasing concentrations of NaCl as indicated. B Immunoblot analysis of HIF-1α immunoprecipitates and whole cell extracts of normoxic and hypoxic HepG2 cells extracted with 0.4 M NaCl. The same blot was subsequently incubated with the indicated antibodies. HSP90 was detected using a monoclonal anti-HSP90β antibody that detects preferentially but not exclusively HSP90β (see Fig. 6).

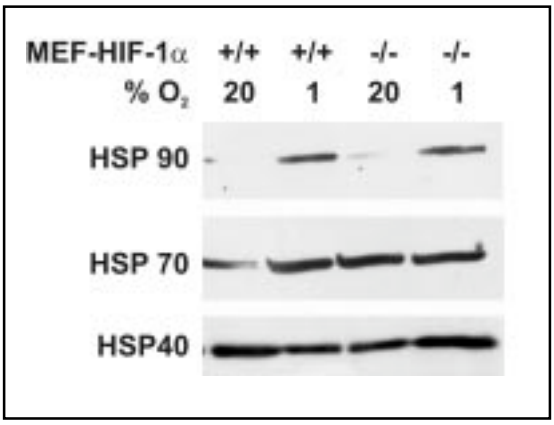

Fig. 5. Hypoxic nuclear accumulation of HSP90 heterocomplex members in wild-type and HIF-1α-deficient cells. MEF-HIF-$1\alpha^{+/+}$ and MEF-HIF-$1\alpha^{-/-}$ cells were kept for 4 hours at 1% O_2. Cells were lysed with NP-40, nuclei were extracted with 0.4 M NaCl, and the extracts analyzed by immunoblotting.

employing an oligonucleotide that contains a conserved HIF-1 DNA-binding site derived from the erythropoietin gene. HIF-1 DNA-binding activity could be observed in nuclear extracts derived from HepG2 cells cultured under hypoxic but not normoxic conditions (Fig. 3B, left). Supershift experiments using a monoclonal anti-HIF-1α antibody confirmed the specificity of the HIF-1 signal. Following the addition of IVTT HSP90, HIF-1 DNA-binding activity decreased. Also the intensity of the constitutive DNA-binding activity, which we previously showed to consist of ATF/CREB family members [38], decreased. However, the non-specific DNA-binding activity was not affected by addition of HSP90 (Fig. 3B), excluding a general suppressive effect on DNA-binding in this assay. A slight suppression of HIF-1 DNA-binding activity by unprimed IVTT extracts (Fig. 3B, right) could be observed, which, however, did not account for the strong suppression by HSP90 cDNA-containing IVTT extracts.

Differential HIF-1α heterocomplex formation in normoxic versus hypoxic cells in vivo

To investigate the interaction between HIF-1α and HSP90 at the cellular level, HepG2 cells were exposed to 1% oxygen for 4 hours. Cytoplasmic and nuclear protein was extracted in a NP-40 buffer containing increasing concentrations of NaCl (0.2 - 1 M). As shown in Fig. 4A, HSP90 co-immunoprecipitated together with HIF-1α in HepG2 extracts, confirming the *in vitro* results. Co-immunoprecipitations worked best in extracts prepared with salt concentrations ranging from 200 to 400 mM NaCl. Since HIF-1α was optimally extracted from the nucleus by NaCl concentrations >200 mM [39] and because 1 M NaCl hindered efficient immunoprecipitation of HIF-1α (Fig. 4A), subsequent immunoprecipitations were performed with 400 mM NaCl. Thus, this represents a compromise between efficient HIF-1α extraction and undisturbed immunoprecipitation.

Co-immunoprecipitations with total cellular extracts derived from normoxic or hypoxic HepG2 cells revealed that besides ARNT and HSP90 other members of the HSP90 heterocomplex, such as HSP70 and probably p23, co-immunoprecipitated with HIF-1α (Fig. 4B, left panel). However, no HSP40 could be detected in this heterocomplex, suggesting a specific composition. Of note, slightly higher levels of HSP90 and HSP70 were detected in HIF-1α precipitates derived from normoxic cells than from hypoxic cells although the HIF-1α levels were much lower in the normoxic cells. In striking contrast, more ARNT could be found in the HIF-1α precipitates

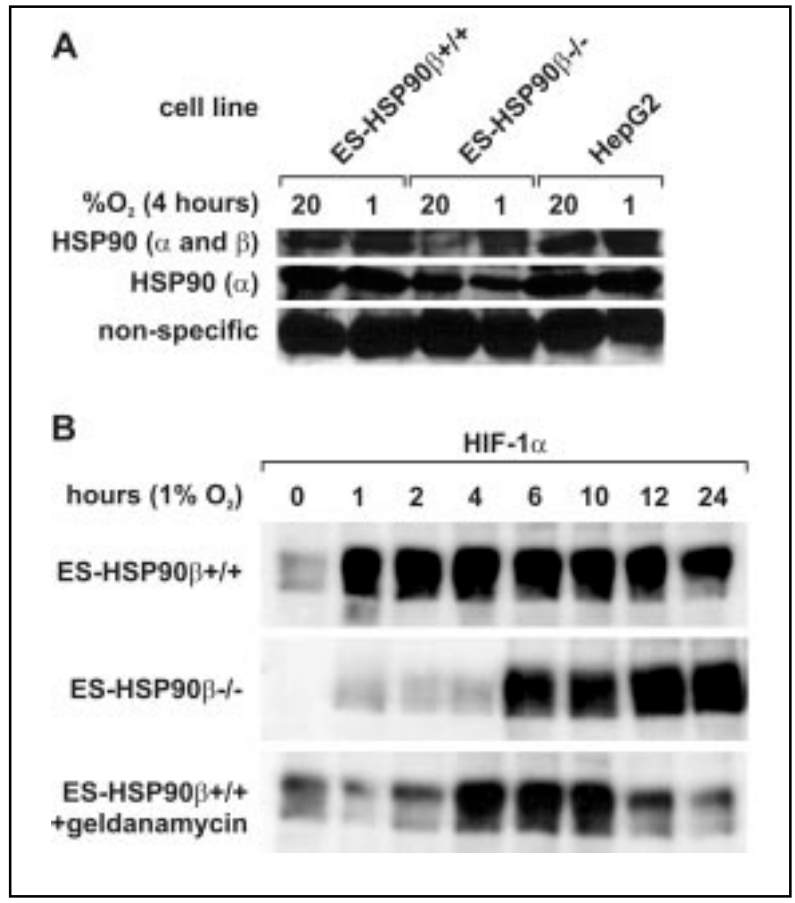

Fig. 6. Kinetics of HIF-1α expression in wild-type and HSP90β-deficient ES cells. A ES-HSP90$^{+/+}$, ES-HSP90$^{-/-}$ and HepG2 cells were exposed to 20% or 1% oxygen for 4 hours. Cell extracts were analysed by immunoblotting, employing a monoclonal anti-HSP90β antibody that detects preferentially but not exclusively HSP90β (top panel). The same blot was subsequently incubated with a polyclonal anti-HSP90α antibody (middle panel). To demonstrate equal protein loading, a non-specific band is shown (bottom panel). B ES-HSP90$^{+/+}$ and ES-HSP90$^{-/-}$ cells were kept for the indicated periods at 1% O_2. 1 μM geldanamycin was added where indicated. HIF-1α protein levels were analyzed by immunoblotting. Note that exposure times were chosen which allowed to visualize both weak and strong signals simultaneously, leading to an over-exposure of the strongest signals.

derived from hypoxic cells than from normoxic cells. The presence of detectable HIF-1α in normoxic cells can be explained by the large amounts of protein extracts (2 mg) used for the co-immunoprecipitations (Fig. 4B, left panel). Indeed, straight immunoblot analysis with less protein extracts (20 μg) allowed detection of HIF-1α in hypoxic but not normoxic cells (Fig. 4B, right panel). These data provide further evidence that the mutually exclusive HIF-1α binding to HSP90 or ARNT found *in vitro* also takes place at the cellular level *in vivo*, and they provide a first link between oxygen concentration and HSP90 heterocomplex function in HIF-1 regulation.

Hypoxic nuclear accumulation of HSP90 is independent of HIF-1α

Displacement of HSP90 from the HIF-1α PAS B interaction site by ARNT is likely to occur in the nucleus since ARNT is a constitutively nuclear protein [39]. However, under normoxic conditions HIF-1α most probably is localized to the cytoplasm [40, 41] where it interacts with HSP90 (see above). We previously demonstrated by immunofluorescence analysis that HSP90 accumulates in the nucleus following hypoxic exposure of the cells without any change in total cellular HSP90 concentrations [26]. One putative mechanism for this finding could be nuclear co-translocation together with HIF-1α. To test this hypothesis, MEFs wild-type or deficient for HIF-1α were analyzed [35]. As shown by immunoblotting, HSP90 but not the heterocomplex members HSP70 and HSP40 accumulated in nuclear extracts of hypoxically stimulated cells (Fig. 5, left part). This effect was independent of the presence of HIF-1α since in HIF-1α-deficient MEFs, HSP90 also accumulated in the nucleus under hypoxic conditions (Fig. 5, right part). Of course, it cannot be excluded from these experiments that HIF-2α or HIF-3α might be responsible for HSP90 nuclear translocation. However, at least regarding target gene induction the other HIF α subunits are not capable of replacing HIF-1α function [35, 42].

HSP90 function is necessary for the rapid hypoxic stabilization of HIF-1α

To elucidate the functional role of HSP90 in hypoxic HIF-1α accumulation and activation, we established a HSP90β-deficient cell model. Therefore, ES cells were isolated from blastocysts recovered from intercrosses of

heterozygous HSP90β-mutant mice. Western blot analysis using a mouse monoclonal anti-HSP90 antibody, known to react preferentially but not exclusively with HSP90β, demonstrated a reduction in the HSP90 signal intensity in HSP90β-deficient ES cells compared with wild-type ES cells under normoxic and hypoxic conditions (Fig. 6A). Subsequently, a rabbit polyclonal anti-HSP90α antibody was applied. Also this antibody resulted in a reduction in the HSP90 signal intensity in HSP90β-deficient ES cells whereas a non-specific cross-reactive signal remained unaffected, demonstrating equal loading between the lanes (Fig. 6A).

Exposure of wild-type ES cells to hypoxia resulted in a rapid and efficient accumulation of HIF-1α, reaching maximal levels after 1 hour of hypoxic exposure (Fig. 6B, top panel). HIF-1α protein became detectable already after 15 minutes of hypoxia (data not shown). In contrast, in HSP90β-deficient ES cells the accumulation of HIF-1α was delayed. Maximal HIF-1α levels were reached only 4 to 6 hours after the onset of hypoxia (Fig. 6B, middle panel). A delayed hypoxic response was also observed in the wild-type ES cells treated with the HSP90 inhibitor geldanamycin (Fig. 6B, bottom panel), confirming the results obtained with the HSP90β-deficient ES cells. Therefore, HSP90 seems to play an important role in the unusually rapid kinetics rather than in the "on-off switch" of hypoxic HIF-1α protein stabilization.

Discussion

Previous studies indicated that HIF-1α interacts with HSP90 under partially artificial conditions [21, 23]. We confirmed and extended these results by demonstrating that endogenous HIF-1α could be co-immunoprecipitated together with the HSP90 heterocomplex from cultured cells. The PAS B domain of HIF-1α was shown to be required for interaction with HSP90. The PAS B domain, and to a minor extend the bHLH domain, is also known to be necessary for HSP90 interaction with the dioxin receptor, another bHLH-PAS transcription factor [43-45]. Interaction with HSP90 retains the inactive dioxin receptor in a ligand-binding competent conformation in the cytoplasm. Upon ligand binding, however, HSP90 is displaced and the dioxin receptor translocates to the nucleus where it interacts with ARNT. Since ligand-binding and HSP90-interaction are two mutually exclusive processes, HSP90 fullfills regulatory mechanism in dioxin receptor signalling.

There is no ligand known capable of interacting with the PAS domain of HIF-1α. However, hypoxia rather than ligand-binding serves as the critical signal for HIF-1α stabilization and activation. Indeed, we showed that under hypoxic conditions, ARNT-binding to HIF-1α increased whereas HSP90- and HSP70-binding decreased. Adding excessive amounts of ARNT competed with HSP90 for HIF-1α binding. Displacement of HSP90 by ARNT from the PAS B domain seems to be a critical step for full HIF-1 activation since addition of HSP90 to the HIF-1 DNA-binding reaction or ectopic overexpression of HSP90 inhibited DNA-binding and transactivation activity. Consistent with our observations, Erbel *et al.* resolved in a recent report the structural basis for the interaction between the PAS B domains of HIF α subunits and ARNT [46].

HSP90 inhibitors like geldanamycin and novobiocin are able to promote proteasome-mediated degradation of HIF-1α in an oxygen- and pVHL-independent manner [23, 26, 27, 32]. Those agents are naturally occurring ansamycin derivatives, exhibiting anti-tumor activities [29-31, 47]. The HSP90 inhibitors are interacting with the nucleotide-binding pocket of HSP90. As a result, the binding of HSP90 to its client proteins is counteracted, followed by the rapid degradation of those instable proteins [48-50]. However, it can not be excluded that these pharmacological HSP90 inhibitors are degrading HIF-1α by indirect and/or unspecific mechanisms. Therefore, we established a genetically HSP90-deficient cell culture model. Vertebrates ubiquitously express two cytosolic HSP90s (HSP90α and HSP90β), the distinct biological functions of which are not completely understood. Mutation of both HSP90 genes is incompatible with cell growth in yeast. Knock-out mice generated from HSP90β-deficient ES cells fail to develop a fetal placental labyrinth and die at embryonic day 9.0/9.5 despite full HSP90α activity [33]. Exposing the HSP90β-deficient ES cells to hypoxic conditions *in vitro* clearly demonstrated that full HSP90 activity is necessary for the rapid hypoxic stabilization of HIF-1α. The presence of both wild-type HSP90α alleles was not sufficient to compensate for the lack of HSP90β function. A similar effect could be provoked by treating wild-type ES cells with geldanamycin, demonstrating that the activity of geldanamycin likely is mediated by HSP90 inhibition.

In summary, a model is emerging in which the HSP90 heterocomplex is bound to the PAS B domain of HIFα subunits and prevents the non-specific degradation of this highly unstable protein by non-pVHL-mediated

ubiquitination and proteasomal degradation. Under normoxic conditions, HSP90 activity thus confers pVHL-specific degradation of HIF-1α and inhibits HIF-1 activity. Under hypoxic conditions, the lack of prolyl hydroxylation prevents pVHL-mediated degradation of HIF-1α and the HSP90 heterocomplex prevents unspecific degradation, accelerating accumulation of HIF-1α. Disruption of HSP90 function delays HIF-1α accumulation. Following nuclear translocation, the high local concentrations of ARNT displace HSP90 from the HIF-1α PAS B domain, without the formation of a ternary complex, allowing the heterodimerization essential for DNA-binding and gene activation.

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